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(54) Title: PRODUCTION OF LUTEIN IN MICROORGANISMS

(57) Abstract: A lycopene ε cyclase from spinach is described, as is production of lutein in microorganisms by expression of the lycopene ε cyclase.

PRODUCTION OF LUTEIN IN MICROORGANISMS

TECHNICAL FIELD

This invention relates to production of lutein in microorganisms.

BACKGROUND

Lutein, a carotenoid produced in many green plants, has been used to pigment animal products such as eggs, foods, drugs, and cosmetics. Shi et al., 1999, Process Biochem., 34:341-347. In addition, lutein has been used for the treatment of age-related macular degeneration, which is the leading cause of blindness in people over 55 years of age. Seddon et al., 1994, J. Am. Med. Assoc., 272:1413-1420.

Typically, lutein is produced by extraction from natural sources. For example, lutein can be extracted from marigold flowers (*Tagetes erecta*) with an organic solvent, then treated with an alkaline solution to provide lutein. Lutein can also be extracted from green vegetables such as kale, spinach, broccoli, green beans, and collard greens. Alternatively, algae producing lutein can be dried and used directly.

SUMMARY

The present invention is based on the identification of a nucleic acid encoding a lycopene ε cyclase from spinach and its use for producing lutein in microorganisms that do not naturally produce or that produce small amounts of lutein. Microorganisms of the present invention can be used as nutritional supplements or as sources of lutein for food products or therapeutics purposes.

In one aspect, the invention features an isolated nucleic acid having at least 75% sequence identity to the nucleotide sequence of nucleotides 264-1817 of SEQ ID NO:5 or to a fragment of the nucleic acid of SEQ ID NO:5 at least about 34 contiguous nucleotides in length. The nucleic acid can have at least 80%, 85%, 90%, or 95% sequence identity to the nucleotide sequence of nucleotides 264-1817 of SEQ ID NO:5 or to the fragment of the nucleic acid of SEQ ID NO:5 at least about 34 contiguous nucleotides in length. An expression vector of the invention can include such a nucleic acid operably linked to an expression control element.

The invention also features an isolated nucleic acid encoding a lycopene ε cyclase polypeptide at least 75% identical to the amino acid sequence of SEQ ID NO:6 and an isolated polypeptide having at least 85% sequence identity to the amino acid sequence of SEQ ID NO:6. The isolated polypeptide can have at least 95% or 99% sequence identity to the amino acid sequence of SEQ ID NO:6.

In another aspect, the invention features a microorganism that produces detectable amounts of lutein, wherein the microorganism includes an exogenous nucleic acid encoding a lycopene ε cyclase. A single polypeptide can convert α-carotene to lutein in the microorganism. The single polypeptide can be exogenous or endogenous to the microorganism. The microorganism may naturally produce β-carotene, hydroxylated derivatives of β-carotene such as zeaxanthin, or lycopene. The microorganism can be a species from a genus selected from the group consisting of *Pantoea, Escherichia,* and *Rhodobacter*. The *Rhodobacter* species can be *R. sphaeroides* or *R. capsulatus*. The *Escherichia* species can be *E. vulneris*. The *Pantoea* species can be *P. stewartii*. The microorganism can be a membranous bacteria.

In yet another aspect, the invention features a microorganism that includes biosynthetic enzymes (geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene dehydrogenase, lycopene β-cyclase, lycopene ε cyclase, and β-carotene hydroxylase). Each of the biosynthetic enzymes is encoded by an exogenous nucleic acid, and expression of the biosynthetic enzymes in the microorganism produces lutein. The microorganism does not naturally produce carotenoids.

A microorganism that produces detectable amounts of zeaxanthin and lutein also is featured. The microorganism includes a first exogenous nucleic acid encoding a lycopene ε cyclase operably linked to a first inducible expression control element, wherein α-carotene is converted to lutein in the microorganism by a single polypeptide, and wherein inducible expression of the first exogenous nucleic acid regulates the ratio of zeaxanthin to lutein in the microorganism. The microorganism also can include a second exogenous nucleic acid encoding a lycopene β cyclase operably linked to a second inducible expression control element, wherein inducible expression of the first and the second exogenous nucleic acids regulates the ratio of zeaxanthin to lutein in the microorganism.

The invention also features a method of producing lutein. The method includes expressing lycopene ε cyclase in a microorganism, wherein the microorganism includes an exogenous nucleic acid encoding the lycopene ε cyclase and extracting lutein from the microorganism. A single polypeptide can convert α-carotene to lutein in the microorganism.

A method of producing a feed supplement also is featured. The method includes expressing lycopene ε cyclase in a microorganism, wherein the microorganism includes an exogenous nucleic acid encoding the lycopene ε cyclase; and drying the microorganism to produce the feed supplement. A single polypeptide can convert α-carotene to lutein in the microorganism.

In yet another aspect, the invention features a method of producing an enhanced food product. The method includes expressing lycopene ε cyclase in a microorganism, wherein the microorganism comprises an exogenous nucleic acid encoding the lycopene ε cyclase; extracting lutein from the microorganism; and adding the lutein to a food product to obtain the enhanced food product.

The invention features an algal cell, wherein the algal cell includes an exogenous nucleic acid encoding a lycopene ε cyclase, and wherein the algal cell produces detectable amounts of lutein.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 is a schematic that depicts the biosynthetic pathway for production of lutein and zeaxanthin.

FIG 2 is the nucleotide sequence of the spinach lycopene ε cyclase (SEQ ID NO:5). The start (ATG) and stop codons (TAA) are bolded and underlined. The coding sequence for the complete lycopene epsilon cyclase gene is from bp 264-1817.

FIG 3 is the amino acid sequence of the spinach lycopene ε cyclase (SEQ ID NO:6).

FIG 4 is a chromatogram of carotenoid extracts from *Escherichia vulneris*. Panels A and B are lutein and zeaxanthin standards, respectively. Panel C is a chromatogram of a carotenoid extract from a negative control (*E. vulneris* transformed with an empty vector). Panel D is a chromatogram of a carotenoid extract from *E. vulneris* transformed with the spinach lycopene ε cyclase.

FIG 5 is a chromatogram of carotenoid extracts from *P. stewartii*. Panel A is a chromatogram of lutein and zeaxanthin standards. Panels B and C are chromatograms of carotenoid extracts from *P. stewartii* cells transformed with pPRONde-LEC after induction (B) or under normal culture conditions (C). Panels D and E are chromatograms of carotenoid extracts from *P. stewartii* cells that were transformed with pPRONde (without insert) after induction (D) or under normal culture conditions (E).

DETAILED DESCRIPTION

Lycopene ε Cyclase Nucleic Acids

The invention features nucleic acids having at least 75% sequence identity, e.g., 80%, 85%, 90%, 95%, or 99% sequence identity, to the nucleic acid sequence of nucleotides 264-1817of SEQ ID NO:5, or fragments of the nucleic acid of SEQ ID NO:5 that are at least about 34 nucleotides in length. Generally, percent sequence identity is calculated by determining the number of matched positions in aligned nucleic acid sequences, dividing the number of matched positions by the total number of aligned nucleotides, and multiplying by 100. A matched position refers to a position in which identical nucleotides occur at the same position in aligned nucleic

acid sequences. The total number of aligned nucleotides refers to the number of nucleotides from SEQ ID NO:5 that are being aligned. Nucleic acid sequences can be aligned by visual inspection or by using sequence alignment software. For example, the Clustal algorithm of MEGALIGN® (DNASTAR, Madison, WI, 1997) sequence alignment software can be used. In this method, sequences are grouped into clusters by examining the distances between all pairs. Clusters are aligned as pairs, then as groups. A gap penalty of 100 and a gap length penalty of 2 are used in the alignments.

As used herein, "isolated" refers to a sequence corresponding to part or all of a nucleic acid encoding a lycopene ε cyclase polypeptide, but free of sequences that normally flank one or both sides of the wild-type nucleic acid in a plant genome. An isolated nucleic acid can be, for example, a DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a DNA molecule that exists as a separate molecule (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid. A nucleic acid existing among hundreds to millions of other nucleic acids within, for example, cDNA libraries or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not to be considered an isolated nucleic acid.

Isolated nucleic acid molecules of the invention are at least about 34 nucleotides in length. For example, the nucleic acid molecule can be about 34 to 45, 40-50, 50-100, or greater than 150 nucleotides in length, e.g., 200-300, 300-500, or 500-1000 nucleotides in length. Such nucleic acids, whether protein encoding or not, can be used as probes, primers, and diagnostic reagents. In some embodiments, the isolated nucleic acid molecules encode a full-length lycopene ε cyclase. Nucleic acid molecules can be DNA or RNA, linear or circular, and in sense or antisense orientation.

Isolated nucleic acids within the scope of the invention can be obtained using any method including, without limitation, common molecular cloning and chemical nucleic acid synthesis techniques. Polymerase chain reaction (PCR) techniques can be used to produce nucleic acid molecules of the invention. PCR refers to a procedure or technique in which target nucleic acids are amplified. Sequence information from the ends of the region of interest or beyond typically is employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers are typically 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, Ed. by Dieffenbach, C. and Dveksler, G., Cold Spring Harbor Laboratory Press, 1995. When using RNA as a source of template, reverse transcriptase can be used to synthesize complimentary DNA (cDNA) strands.

Nucleic acids encoding lycopene ε cyclase polypeptides also can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector.

Isolated nucleic acids of the invention also can be obtained by mutagenesis. For example, an isolated nucleic acid that shares identity with a sequence set forth in SEQ ID NO: 5 can be mutated using common molecular cloning techniques (e.g., site-directed mutagenesis). Possible mutations include, without limitation, deletions, insertions, and substitutions, as well as combinations of deletions, insertions, and substitutions. Alignments of nucleic acids of the invention with other known sequences encoding carotenoid enzymes can be used to identify positions to modify. For example, alignment of the nucleotide sequence of SEQ ID NO:5 with another nucleic acid encoding lycopene ε cyclase provides guidance as to which nucleotides can be substituted, which nucleotides can be deleted, and at which positions

nucleotides can be inserted.

In addition, nucleic acid and amino acid databases (e.g., GenBank®) can be used to obtain an isolated nucleic acid within the scope of the invention. For example, any nucleic acid sequence having homology to a sequence set forth in SEQ ID NO: 5 or any amino acid sequence having homology to a sequence set forth in SEQ ID NO: 6 can be used as a query to search GenBank®.

Furthermore, nucleic acid hybridization techniques can be used to obtain an isolated nucleic acid within the scope of the invention. Briefly, any nucleic acid having some homology to a sequence set forth in SEQ ID NO: 5 can be used as a probe to identify a similar nucleic acid by hybridization under conditions of moderate to high stringency. Moderately stringent hybridization conditions include hybridization at about 42°C in a hybridization solution containing 25 mM KPO_4 (pH 7.4), 5X SSC, 5X Denhart's solution, 50 μg/mL denatured, sonicated salmon sperm DNA, 50% formamide, 10% Dextran sulfate, and 1-15 ng/mL probe (about $5x10^7$ cpm/μg), and wash steps at about 50°C with a wash solution containing 2X SSC and 0.1% SDS. For high stringency, the same hybridization conditions can be used, but washes are performed at about 65°C with a wash solution containing 0.2X SSC and 0.1% SDS.

Once a nucleic acid is identified, the nucleic acid then can be purified, sequenced, and analyzed to determine whether it is within the scope of the invention as described herein. Hybridization can be done by Southern or Northern analysis to identify a DNA or RNA sequence, respectively, that hybridizes to a probe. The probe can be labeled with biotin, digoxygenin, an enzyme, or a radioisotope such as ^{32}P or ^{35}S. The DNA or RNA to be analyzed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe using standard techniques well known in the art. See, for example, sections 7.39-7.52 of Sambrook *et al.*, (1989) Molecular Cloning, second edition, Cold Spring harbor Laboratory, Plainview, NY.

Lycopene ε Cyclase Polypeptides

The present invention also features isolated lycopene ε cyclase polypeptides having at least 85% sequence identity, e.g., 90%, 95%, or 99% sequence identity, to

the amino acid sequence of SEQ ID NO:6 and fragments thereof. An "isolated polypeptide" has been separated from cellular components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60% (e.g., 70%, 80%, 90%, or 95%), by weight, free from proteins and naturally-occurring organic molecules that are naturally associated with it. Percent sequence identity can be determined as described above for nucleic acid molecules. The term "polypeptide" includes any chain of amino acids, regardless of length or post-translational modification. Polypeptides that are at least 85% identical to the amino acid sequence of SEQ ID NO:6 can retain the function of lycopene ε cyclase; i.e., such polypeptides are able to catalyze the formation of ε rings on carotenoids such as lycopene. In general, conservative amino acid substitutions, i.e., substitutions of similar amino acids, are tolerated without affecting protein function. Similar amino acids are those that are similar in size and/or charge properties. For example, aspartate and glutamate, and isoleucine and valine, are pairs of similar amino acids. Similarity between amino acid pairs is well known in the art and can be assessed in a number of ways. Dyahoff et al., Atlas of Protein Sequence and Structure, 1978, 5(3):345-352, is a representation of the skill of the art and provides frequency tables for amino acid substitutions that can be employed as a measure of amino acid similarity.

An isolated lycopene ε cyclase polypeptide can be obtained, for example, by extraction from a natural source (e.g., a plant cell), chemical synthesis, or by production in a host. For example, a lycopene ε cyclase polypeptide of the present invention can be produced by ligating a nucleic acid molecule encoding the polypeptide into a nucleic acid construct such as an expression vector, and transforming a bacterial or eukaryotic host cell with the expression vector. In general, nucleic acid constructs include expression control elements operably linked to a nucleic acid sequence encoding a lycopene ε cyclase polypeptide. Expression control elements do not typically encode a gene product, but instead affect the expression of the nucleic acid sequence. As used herein, "operably linked" refers to connection of the expression control elements to the nucleic acid sequence in such a way as to permit expression of the nucleic acid sequence. Expression control elements can include, for example, promoter sequences, enhancer sequences, response elements, polyadenylation sites, or inducible elements. Non-limiting examples of promoters include the *puf* promoter from *Rhodobacter sphaeroides* (GenBank Accession No.

E13945), the *nifHDK* promoter from *R. sphaeroides* (GenBank Accession No. AF031817), and the *fliK* promoter from *R. sphaeroides* (GenBank Accession No. U86454).

In bacterial systems, a strain of *E. coli* such as BL-21 can be used. Suitable *E. coli* vectors include, but are not limited to, the pGEX series of vectors that produce fusion proteins with glutathione S-transferase (GST). Transformed *E. coli* are typically grown exponentially then stimulated with isopropylthiogalactopyranoside (IPTG) prior to harvesting. In general, such fusion proteins are soluble and can be purified easily from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites such that the cloned target gene product can be released from the GST moiety.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express lycopene ε cyclase polypeptides. A nucleic acid encoding a lycopene ε cyclase polypeptide can be cloned into, for example, a baculoviral vector such as pBlueBac (Invitrogen, San Diego, CA) and then used to co- transfect insect cells such as *Spodoptera frugiperda* (Sf9) cells with wild-type DNA from *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV). Recombinant viruses producing the lycopene ε cyclase polypeptides can be identified by standard methodology. Alternatively, a nucleic acid encoding a lycopene ε cyclase polypeptide can be introduced into a SV40, retroviral, or vaccinia based viral vector and used to infect suitable host cells.

A polypeptide within the scope of the invention can be "engineered" to contain an amino acid sequence that allows the polypeptide to be captured onto an affinity matrix. For example, a tag such as c-myc, hemagglutinin, polyhistidine, or Flag™ tag (Kodak) can be used to aid polypeptide purification. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino termini. Other fusions that could be useful include enzymes that aid in the detection of the polypeptide, such as alkaline phosphatase.

Agrobacterium-mediated transformation, electroporation and particle gun transformation can be used to transform plant cells. Illustrative examples of transformation techniques are described in U.S. Patent No. 5,204,253 (particle gun) and U.S. Patent No. 5,188,958 (*Agrobacterium*). Transformation methods utilizing

the Ti and Ri plasmids of *Agrobacterium spp.* typically use binary type vectors. Walkerpeach, C. et al., in Plant Molecular Biology Manual, S. Gelvin and R. Schilperoort, eds., Kluwer Dordrecht, C1:1-19 (1994). If cell or tissue cultures are used as the recipient tissue for transformation, plants can be regenerated from transformed cultures by techniques known to those skilled in the art.

Engineered Microorganisms

A nucleic acid encoding lycopene ε cyclase polypeptide can be expressed in microorganisms so that detectable amounts of lutein are produced. As used herein, "detectable" refers to the ability to detect lutein and esters thereof using standard analytical methodology. In general, lutein can be extracted with an organic solvent such as acetone and detected by an absorption scan from 400-500 nm in the same organic solvent. In some cases, it is desirable to back-extract with a second organic solvent, such as hexane. The maximal absorbance of lutein depends on the solvent that it is in. For example, in acetone, the maximal absorbance of lutein is at 451 nm, while maximal absorbance of zeaxanthin is at 454 nm. In hexane, the maximal absorbance of lutein and zeaxanthin is 446 nm and 450 nm, respectively.

High performance liquid chromatography coupled to mass spectrometry also can be used to detect lutein. Two reverse phase columns that are connected in series can be used with a solvent gradient of water and acetone. The first column is a C30 specialty column designed for carotenoid separation (e.g., YMCä Carotenoid S3m; 2.0 x 150 mm, 3mm particle size; Waters Corporation, PN CT99S031502WT) followed by a C8 Xterraä MS column (e.g., Xterraä MS C8; 2.1 x 250 mm, 5mm particle size; Waters Corporation, PN 186000459).

Detectable amounts of lutein include 10μg/g dry cell weight (dcw) and greater. For example, about 10 to 100,000μg/g dcw, about 100 to 60,000μg/g dcw, about 500 to 30,000μg/g dcw, about 1000 to 20,000 μg/g dcw, or about 5,000 to 15,000 μg/g dcw. With respect to algae or other plants or organisms that produce lutein, "detectable amount of lutein" is an amount that is detectable over the endogenous level in the plant or organism.

Upon expression of lycopene ε cyclase in a microorganism, the lycopene ε cyclase converts lycopene to δ-carotene by formation of an ε-ring. Subsequently, δ-

carotene is converted to α-carotene by lycopene β-cyclase. As demonstrated herein, many microorganisms surprisingly can use a single hydroxylase to convert α-carotene to lutein. Without being bound by a particular mechanism, it is thought that this hydroxylase can hydroxylate the β or ε-rings of α-carotene, the β-ring of α-cryptoxanthin, or the ε -ring of physoxanthin. See, "Key to Carotenoids", Edited by Straub, O., Birkhäuser Verlag, 1987, p. 34-36. In plants, two hydroxylases are required, one that is specific for the β-ring and one that is specific for the ε-ring. See, for example, Pogson et al., 1996, Plant Cell, 8:1627-1639. The single hydroxylase may be endogenous (i.e., a nucleic acid or polypeptide in the naturally-occurring microorganism) or exogenous to the microorganism.

Microorganisms that are suitable for producing lutein may or may not naturally produce carotenoids, and include prokaryotic and eukaryotic microorganisms, such as bacteria, yeast, and fungi. In particular, yeast such as *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) and *Saccharomyces cerevisiae*, fungi such as *Neurospora crassa*, *Phycomyces blakesleeanus*, *Blakeslea trispora*, and *Aspergillus sp*, Archaea bacteria such as *Halobacterium salinarium*, and Eubacteria including *Pantoea* species (formerly called *Erwinia*) such as *P. stewartii*, flavobacteria species such as *Xanthobacter autotrophicus* and *Flavobacterium multivorum*, *Rhodobacter* species such as *R. sphaeroides* or *R. capsulatus*, and *Escherichia* species such as *E. coli* and *E. vulneris* can be used. Eubacteria are particularly useful. In addition, algae can be used, including *Haematococcus pluvialis*, *Dunaliella salina*, *Chlorella protothecoides*, and *Neospongiococcum excentrum*. Algae produce minor amounts of lutein.

It is noted that bacteria can be membranous or non-membranous bacteria. The term "membranous bacteria" as used herein refers to any naturally-occurring, genetically modified, or environmentally modified bacteria having an intracytoplasmic membrane. An intracytoplasmic membrane can be organized in a variety of ways including, without limitation, vesicles, tubules, thylakoid-like membrane sacs, and highly organized membrane stacks. Any method can be used to analyze bacteria for the presence of intracytoplasmic membranes including, without limitation, electron microscopy, light microscopy, and density gradients. See, e.g., Chory et al., (1984) J. Bacteriol., 159:540-554; Niederman and Gibson, Isolation and Physiochemical Properties of Membranes from Purple Photosynthetic Bacteria. In:

The Photosynthetic Bacteria, Ed. By Roderick K. Clayton and William R. Sistrom, Plenum Press, pp. 79-118 (1978); and Lueking et al., (1978) J. Biol. Chem., 253: 451-457.

Examples of membranous bacteria that can be used include, without limitation, Purple Non-Sulfur Bacteria, including bacteria of the Rhodospirillaceae family such as those in the genus *Rhodobacter* (e.g., *R. sphaeroides* and *R. capsulatus*), the genus *Rhodospirillum*, the genus *Rhodopseudomonas*, the genus *Rhodomicrobium*, and the genus *Rhodopila*. The term "non-membranous bacteria" refers to any bacteria lacking intracytoplasmic membrane. Membranous bacteria can be highly membranous bacteria. The term "highly membranous bacteria" as used herein refers to any bacterium having more intracytoplasmic membrane than *R. sphaeroides* (ATCC 17023) cells have after the *R. sphaeroides* (ATCC 17023) cells have been (1) cultured chemoheterotrophically under aerobic condition for four days, (2) cultured chemoheterotrophically under anaerobic for four hours, and (3) harvested. Aerobic culture conditions include culturing the cells in the dark at 30°C in the presence of 25% oxygen. Anaerobic culture conditions include culturing the cells in the light at 30°C in the presence of 2% oxygen. After the four hour anaerobic culturing step, the *R. sphaeroides* (ATCC 17023) cells are harvested by centrifugation and analyzed.

Microorganisms containing an isolated nucleic acid of the invention are not required to express the isolated nucleic acid. Isolated nucleic acids can be integrated into the genome of the microorganism or maintained in an episomal state. In other words, microorganisms can be stably or transiently transfected with an isolated nucleic acid of the invention.

Any method can be used to introduce an isolated nucleic acid into a microorganism. In fact, many methods for introducing nucleic acid into cells, whether *in vivo* or *in vitro*, are well known to those skilled in the art. For example, calcium phosphate precipitation, conjugation, electroporation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer are common methods that can be used to introduce nucleic acid molecules into cells. In addition, naked DNA can be delivered directly to cells *in vivo* as describe elsewhere (U.S. Patent Nos. 5,580,859 and 5,589,466).

Any method can be used to identify microorganisms that contain an isolated nucleic acid within the scope of the invention. For example, PCR and nucleic acid hybridization techniques such as Northern and Southern analysis can be used. In some cases, immunohistochemistry and biochemical techniques can be used to determine if a microorganism contains a particular nucleic acid by detecting the expression of a polypeptide encoded by that particular nucleic acid. For example, the polypeptide of interest can be detected with an antibody having specific binding affinity for that polypeptide, which indicates that that cell not only contains the introduced nucleic acid but also expresses the encoded polypeptide. Enzymatic activities of the polypeptide of interest also can be detected or an end product (e.g., lutein) can be detected as an indication that the microorganism contains the introduced nucleic acid and expresses the encoded polypeptide from that introduced nucleic acid.

The microorganisms described herein can contain a single copy or multiple copies (e.g., about 5, 10, 20, 35, 50, 75, 100 or 150 copies), of a particular exogenous nucleic acid. All non-naturally-occurring nucleic acids are considered an exogenous nucleic acid once introduced into the cell. The term "exogenous" as used herein with reference to a nucleic acid and a particular cell refers to any nucleic acid that does not originate from that particular cell as found in nature. Nucleic acid that is naturally-occurring also can be exogenous to a particular cell. For example, an entire operon that is isolated from a bacteria is an exogenous nucleic acid with respect to a second bacteria once that operon is introduced into the second bacteria. For example, a bacterial cell (e.g., *Rhodobacter*) can contain about 50 copies of an exogenous nucleic acid of the invention. In addition, the cells described herein can contain more than one particular exogenous nucleic acid. For example, a bacterial cell can contain about 50 copies of exogenous nucleic acid X as well as about 75 copies of exogenous nucleic acid Y. In these cases, each different nucleic acid can encode a different polypeptide having its own unique enzymatic activity. For example, a bacterial cell can contain two different exogenous nucleic acids such that a high level of lutein is produced. In addition, a single exogenous nucleic acid can encode one or more polypeptides.

Depending on the microorganism and the metabolites present within the microorganism, one or more of the following enzymes may be expressed in the

microorganism in addition to lycopene ε cyclase: geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene dehydrogenase, lycopene β cyclase, and β-carotene hydroxylase. Geranylgeranyl pyrophosphate synthase condenses one farnesyl pyrophosphate (FPP) and one isopentenyl pyrophosphate (IPP) molecule to form geranylgeranyl pyrophosphate (GGPP). Alternatively, one could utilize a multifunctional GGPP synthase that can directly prepare GGPP from IPP and dimethylallyl pyrophosphate (DMAPP). Phytoene synthase condenses two GGPP molecules to form phytoene. Phytoene dehydrogenase (also known as phytoene desaturase) performs four successive desaturations to convert phytoene to lycopene (see FIG 1). Lycopene ε cyclase converts lycopene to δ-carotene, lycopene β cyclase converts δ-carotene to α-carotene, and β-carotene hydroxylase converts α-carotene to lutein. The genes encoding such enzymes have been cloned and sequenced from many organisms. See, for example, Genbank Accession No. Y15112 for the sequence of carotenoid biosynthesis genes of *Paracoccus marcusii*; Genbank Accession No. D58420 for the carotenoid biosynthesis genes of *Agrobacterium aurantiacum*; Genbank Accession No. M87280 M99707 for the sequence of carotenoid biosynthesis genes of *Erwinia herbicola*; and Genbank Accession No. U62808 for carotenoid biosynthesis genes of *Flavobacterium* sp. Strain R1534.

For example, a microorganism that naturally produces carotenoids such as β-carotene or hydroxylated derivatives of β-carotene (e.g., zeaxanthin) can be used. Typically, β-carotene derivatives are hydroxylated on a ring and more specifically, at the 3/3' position of the ring. Such microorganisms have the required enzymes to produce lutein after lycopene ε cyclase is expressed and is producing δ-carotene. Other suitable microorganisms naturally produce carotenoids such as lycopene, but do not produce zeaxanthin. Additional enzymes are required in these organisms for production of lutein, including lycopene β-cyclase and a hydroxylase that can convert α-carotene to lutein. In microorganisms that naturally produce FPP, but do not naturally produce carotenoids, it is necessary to introduce the enzymes that produce the substrate for lycopene ε cyclase and the enzymes to convert δ-carotene to lutein. These enzymes include geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene dehydrogenase, lycopene β cyclase, and β-carotene hydroxylase.

Control of the Ratio of Zeaxanthin to Lutein

In microorganisms that produce zeaxanthin, the ratio of zeaxanthin to lutein can be manipulated. For example, expression of lycopene β cyclase and/or lycopene ε cyclase can be controlled by an inducible promoter or by use of constitutive promoters of different strengths. As used herein, "inducible" refers to both up-regulation and down regulation. An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer, the DNA sequences or genes will not be transcribed. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, phenolic compound, or a physiological stress imposed directly by heat, cold, salt, or toxic elements, or indirectly through the action of a pathogen or disease agent such as a virus. The inducer also can be an illumination agent such as light, darkness and light's various aspects, which include wavelength, intensity, fluorescence, direction, and duration. Examples of inducible promoters include the lac system and the tetracycline resistance system from *E.coli*. In one version of the Lac system, expression of lac operator-linked sequences is constitutively activated by a LacR-VP16 fusion protein and is turned off in the presence of IPTG. In another version of the Lac system, a lacR-VP16 variant is used that binds to lac operators in the presence of IPTG, which can be enhanced by increasing the temperature of the cells. The lac promoter also can contain the recognition site for AraC, the repressor-inducer from the BAD promoter of the *ara* operon. A plasmid containing the lac promoter and an AraC recognition site (e.g., pPROLar), is available commercially from Clontech Laboratories Inc (Palo Alto, CA). See, Lutz and Bujard, Nucleic Acids Res. (1997) 25(6):12-3-1210.

Components of the tetracycline (Tc) resistance system also have been used to regulate gene expression. For example, the Tet repressor (TetR), which binds to tet operator sequences in the absence of tetracycline and represses gene transcription, can be used to repress transcription from a promoter containing tet operator sequences. TetR also can be fused to the activation domain of VP 16 to create a tetracycline-controlled transcriptional activator (tTA), which is regulated by tetracycline in the same manner as TetR, i.e., tTA binds to tet operator sequences in the absence of tetracycline but not in the presence of tetracycline. Thus, in this system, in the

continuous presence of Tc, gene expression is kept off, and to induce transcription, Tc is removed.

Alternative methods of controlling the ratio of zeaxanthin to lutein include using enzyme inhibitors to regulate the activity levels of lycopene β or ε cyclases. Examples of lycopene cyclase inhibitors include 2-(4-chlorophenylthio)-triethylamine, α-picoline, nicotine, and imidazoles. Specificity of such inhibitors for lycopene β or ε cyclases can be confirmed using extracts containing both lycopene β and ε cyclase activity.

Production of Lutein in Microorganisms

Typically, lutein is produced by culturing an engineered microorganism of the invention in culture medium and extracting lutein from the cultured microorganisms. In general, the culture media and/or culture conditions are such that the microorganisms grow to an adequate density and produce lutein efficiently. For large-scale production processes, the following methods can be used. First, a large tank (e.g., a 100 gallon, 200 gallon, 500 gallon, or more tank) containing appropriate culture medium with, for example, a glucose carbon source is inoculated with a particular microorganism. After inoculation, the microorganisms are incubated to allow biomass to be produced. Once a desired biomass is reached, the broth containing the microorganisms can be transferred to a second tank. This second tank can be any size. For example, the second tank can be larger, smaller, or the same size as the first tank. Typically, the second tank is larger than the first such that additional culture medium can be added to the broth from the first tank. In addition, the culture medium within this second tank can be the same as, or different from, that used in the first tank. For example, the first tank can contain medium with xylose, while the second tank contains medium with glucose. In addition, inducers can be added to the culture medium to enhance production of lutein.

Once transferred, the microorganisms can be incubated to allow for the production of lutein. Once produced, any method can be used to isolate the desired compound. For example, if the microorganism releases lutein into the broth, then common separation techniques can be used to remove the biomass from the broth, and common isolation procedures (e.g., extraction, distillation, and ion-exchange

procedures) can be used to obtain lutein from the microorganism-free broth. In addition, lutein can be isolated while it is being produced, or it can be isolated from the broth after the product production phase has been terminated. If the microorganism retains lutein, the biomass can be collected and lutein can be released by treating the biomass or lutein can be extracted directly from the biomass. Once extracted, lutein can be, for example, formulated into therapeutic compositions or can be incorporated into a food product (e.g., mixed with a food product) to produce an enhanced food product.

Alternatively, the biomass can be collected and dried, without extracting the lutein. The biomass then can be formulated for human consumption (e.g., as a dietary supplement) or as an animal feed. One or more components can be added to the biomass before or after drying, including vitamins, other carotenoids, antioxidants such as ethoxyquin, vitamin E, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or ascorbyl palmitate, vegetable oils such as corn oil, safflower oil, sunflower oil, or soybean oil, and an edible emulsifier, such as soy bean lecithin or sorbitan esters. Addition of antioxidants and vegetable oils can help prevent degradation of lutein during processing (e.g., drying), shipment, and storage of the composition.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 – Cloning of Lycopene ε Cyclase from Spinach: Lycopene ε cyclase was identified from spinach (*Spinacea oleracea*) by amplifying a PCR product from a lambda ZAP II cDNA spinach library (Stratagene, La Jolla, CA). Degenerate oligonucleotides with the sequence: 5'-WAG AAA YCC YTG CCA CAT CCA TTT TG-3', wherein W= A or T and Y= C or T (SEQ ID NO:1) and 5'-GGC CGT GCY TAT GGA AGA GTT AGT C-3' (SEQ ID NO:2) were used to prime the PCR reaction. The nucleotide sequences of SEQ ID NOS: 1 and 2 align with nucleotides 1384-1407 and 508-532, respectively, of the open reading frame of the lycopene ε cyclase that was identified. These primers were based on homologous regions from the tomato (*Lycopersicon esculentum*) and *Arabidopsis thaliana*

lycopene ε cyclase genes that did not show high homology to regions from the tomato and *A. thaliana* lycopene β cyclase genes. An enriched pool of products was produced by amplifying the cDNA spinach library with the degenerate primers by incubating at 96°C for 5 minutes, followed by 25 cycles of denaturation at 96°C for 30 seconds, annealing at 45°C for 60 seconds, and extension at 72°C for 2 minutes, followed by incubation at 72°C for 10 minutes. A portion of the reaction (10μl) was removed and reamplified with the degenerate primers (additional 25 cycles of PCR). An 899 bp PCR product was purified and sequenced from the second amplification. Non-degenerate oligonucleotides were synthesized based on the sequence of the 899 bp PCR product and used to generate an 800 bp probe to screen the cDNA library. The primers that were synthesized have the following sequences: 5'-GCA TAT GGA GCA GCT TCA GGT A-3' (SEQ ID NO:3, nucleotides 951-972 of SEQ ID NO:5) and 5'-GGA GGT GCG GAA GAA TGT CCT GAT-3' (SEQ ID NO:4, nucleotides 1611-1634 of SEQ ID NO:5). Similar PCR reactions were used as described above, except that 30 cycles were used, annealing was performed at 57°C, and extension was performed for 90 seconds. A digoxigenin (DIG) labeling system from Roche Biosytems (Basel, Switzerland) was used to produce probe that was labeled by incorporation of DIG-11-dUTP during PCR.

The DIG labeled probe was purified and used to screen the cDNA spinach library. Hybridization conditions included DIG Easy Hyb solution (Boehringer Mannheim) and incubation at 42°C for two hours, followed by two washes in 2X SSC, 0.1% SDS (w/v) for 5 minutes each at room temperature and two washes in 0.5X SSC, 0.1% SDS (w/v) for 15 minutes each at 68°C with gentle agitation. Detection of probe was per manufacturer's instructions (Boehringer Mannheim). Plaques that hybridized to the probe were picked. Phage particles were eluted and re-plated to isolate individual plaques. The individual plaques were re-probed with the same DIG-labeled probe to ensure that the plaques were pure. From the purified lambda ZAP plaque, the pBLUESCRIPT (pBS) vector that was embedded in the phage DNA was excised. This excised pBS carried the cDNA of interest. The plasmid was purified and used to transform *E. coli* DH10B cells via electroporation. Plasmid DNA was isolated from colonies and sequenced.

The lycopene ε cyclase cDNA has the nucleotide sequence shown in Figure 2 (SEQ ID NO:5) and encodes a protein having the amino acid sequence shown in

Figure 3 (SEQ ID NO:6). The full-length protein is 517 amino acids in length and has a predicted molecular weight of approximately 58 kDa. The translation start site of the spinach lycopene ε cyclase gene is at nucleotide 264. The spinach lycopene ε cyclase gene has limited homology to lycopene ε cyclase genes from other plants and organisms. For example, the spinach lycopene ε cyclase gene is 66% identical to the lycopene ε cyclase from tomato, 64.6% identical to the *Gentiana lutea* lycopene ε cyclase, and 65.5% identical to the *Arabidopsis thaliana* lycopene ε cyclase gene. The spinach lycopene ε cyclase has 73.2% identity to a partial sequence of the *Dactus carota* lycopene ε cyclase (alignment of nucleotides 664 to 1160 of spinach lycopene ε cyclase).

Example 2 – Production of Lutein in *Pantoea stewartii*: The spinach lycopene ε cyclase was expressed in *Pantoea stewartii* (formerly called *Erwinia*), which was obtained from the American Type Culture Collection (Accession No. 8200).

Electrocompetent *P. stewartii* cells were prepared by culturing a 5% inoculum of *P. stewartii* cells in 50 ml of LB at 26°C with agitation (250 rpm) until an OD_{590} of 0.825 was reached. The bacteria were washed in 50 ml of 10mM HEPES, pH 7.0, and centrifuged for 10 minutes at 10,000xg. The wash was repeated with 25 ml of 10mM HEPES, pH 7.0, followed by the same centrifugation protocol. The cells then were washed once in 25 ml of 10% glycerol. Following centrifugation, the cells were resuspended in 500µl of 10% glycerol. Aliquots were frozen and kept at -80°C until use.

The purified pBS vector carrying the nucleic acid encoding the spinach lycopene ε cyclase (pBS:spLEC) was used to transform the *P. stewartii* cells by electroporation. The control was *P. stewartii* transformed with the pBS vector without the spinach lycopene ε cyclase cDNA. Electroporation conditions were 25 kV/cm, 200 Ohms, and 25 µfarads. Liquid cultures were grown and carotenoids were extracted by the following procedure. Approximately 20 ml cultures of *P. stewartii* were centrifuged at 10,000xg for 5 minutes to pellet the cells. Supernatants were decanted and the cells were washed with 10ml of sterile, distilled water and centrifuged again. Cells were resuspended in 3 ml of acetone, scraped and crushed with a spatula, then kept in the dark for twenty minutes. After twenty minutes, the acetone extract was removed and placed in a separate tube. Approximately 1 ml of

hexane was added to the tube, and the mixture was vortexed. Water (3 ml) was added to the tube and re-vortexed. The tube was centrifuged for two minutes at 1,500xg to separate the hexane and acetone/water layers. The absorbance of the hexane layer was measured in a scan from 400-500nm. An approximate 4nm shift in the maximum absorbance was observed between transformants expressing lycopene ε cyclase and the control cells (447 nm vs 451 nm, respectively), which is indicative of lutein production. Yield was calculated to be ~139.5-289.4μg lutein/g dcw.

Production of lutein was confirmed by HPLC/MS. A Waters 2690 LC System was used with two reversed-phase columns connected in series. The first column was a C30 specialty column designed for carotenoid separation (YMCä Carotenoid S3m; 2.0 x 150 mm, 3mm particle size; Waters Corporation, PN CT99S031502WT) followed by a C8 Xterraä MS column (Xterraä MS C8; 2.1 x 250 mm, 5mm particle size; Waters Corporation, PN 186000459). The columns are thermostated at 25°C. A Binary Gradient was used to separate lutein and zeaxanthin (Mobile Phase A: water. Mobile Phase B: acetone. Flow rate at 0.30 mL/min. Elution program: initial, 90%B; 0-10 min, linear ramp up to 100%B; 10-15 min, hold at 100%B; 15-16 min, ramp down to 90%B; 16-20 min, re-equilibrate to initial conditions). Samples were dissolved in acetone prior to injection and kept cold in the autosampler (dark) at 10°C. Injection volume was 25μl. A Photodiode Array detector, 350-550 nm, was used to detect the separated carotenoids (lutein and zeaxanthin). Under these chromatography conditions, lutein eluted at approximately 5.22 minutes and zeaxanthin eluted at approximately 5.57 minutes. Carotenoid standards were used to identify the peaks. Lutein was obtained from Sigma Chemical Co. (St. Louis, MO) and β-cryptoxanthin and zeaxanthin were obtained from Extrasynthese (France). UV-Vis absorption spectra were used as diagnostic features for the carotenoids as were the molecular ion and fragmentation patterns generated using mass spectrometry. A positive-ion atmospheric pressure chemical ionization mass spectrometer was used, scan range, 400-800 m/z with a quadrupole ion trap.

Example 3 – Production of Lutein in *Escherichia vulneris*: The spinach lycopene ε cyclase was expressed in *Escherichia vulneris*, which was obtained from the American Type Culture Collection (Accession No. 39368).

Electrocompetent *E. vulneris* cells were prepared by culturing a 1% inoculum of *E. vulneris* cells in 50 ml of Nutrient broth from an overnight culture. Cells were incubated ~3 hours to an OD_{600} of ~0.68. The bacteria were washed in 50 ml of 10 mM HEPES (pH 7.0) and centrifuged for 10 minutes at 10,000 x g. The wash was repeated with 25 ml of 10 mM HEPES (pH 7.0), followed by the same centrifugation protocol. The cells then were washed once in 25 ml of 10% glycerol. Following centrifugation, the cells were resuspended in 500 µl of 10% glycerol. Forty µL aliquots were frozen and kept at –80°C until use.

Electrocompetent *E. vulneris* were transformed by the following electroporation procedure: 1 µL of pBS (0.072 µg, without insert) or pBS:SpLEC (0.27 µg, with spinach LEC) were added to 40 µL aliquots of electrocompetent *E. vulneris* and incubated on ice for 3 minutes. Cells were then electroporated at 20 kV/cm, 200 Ohms, 25 µfarads, resuspended in 1 ml SOC, and incubated at 30°C with shaking for 60 minutes. Cells were pelleted at 10,000 x g for 30 seconds and resuspended in 100 µL of fresh SOC. Aliquots of cells (75 µL and 25 µL) were plated onto separate LB + 100 µg/ml Ampicillin plates and allowed to grow overnight at 26°C.

Carotenoid pigments were extracted from *E. vulneris* in the following manner. Single colonies of either *E. vulneris* transformed with pBS or *E. vulneris* transformed with pBS:spLEC were inoculated into 20 ml of Nutrient Broth + 100 µg/ml Ampicillin. Cultures were incubated at 30°C with shaking (250 rpm) for 5 days and harvested by centrifugation at 10,000 x g for 10 min at 4°C. Cell pellets were washed once with 20 ml H_2O and resuspended in 2 ml methanol. The re-suspended cell pellets were incubated in methanol for 60 minutes at 65°C with occasional vortexing.

Cells were pelleted by centrifugation at 10,000 x g for 10 minutes at 4°C and the methanol supernatant was removed to fresh 1.5 ml eppendorf tubes. The extracts were vacuum dried at 20°C in a Speed Vac ISS 110 (Savant Corporation, Holbrook, NY) and resuspended in a 85% methanol / 15% tert-butyl methyl ether mixture.

E. vulneris carotenoid extracts were analyzed by HPLC/MS as described above, except Mobile Phase A was 85% methanol/15% tert-butyl methyl ether and Mobile Phase B was 80% methanol / 20% tert-butyl methyl ether. Under these chromatography conditions, lutein eluted at approximately 5.7 minutes and zeaxanthin eluted at approximately 6.6 minutes. FIG 4 contains a representative

chromatogram of carotenoid extracts from *E. vulneris*. Panels A and B of FIG 4 are chromatograms of lutein and zeaxanthin standards, respectively, while panel C is a chromatogram of a carotenoid extract from a negative control (*E. vulneris* transformed with pBS without insert). Production of lutein in *E. vulneris* cells transformed with pBS:spLEC is shown in panel D of FIG 4. The lutein peak is marked with an arrow in panels A and D. Mass spectral analysis confirmed production of lutein in *E. vulneris* cells transformed with pBS:spLEC.

Example 4 – Control of ratio of lutein and zeaxanthin in *Pantoea stewartii*:

Constructs for regulating the expression of the spinach lycopene ε cyclase were produced from the pPROLarA.122 vector (ClonTech Laboratories, Inc. Palo Alto, CA), which includes a *lac* promoter that contains *lac* operator sequences and the recognition site for AraC, the repressor-inducer from the BAD promoter of the *ara* gene. A *Nde*I restriction site was introduced at bp 132 (nucleotide numbering as described by ClonTech laboratories) of pPROLarA.122 to generate pPRONde. The Stratagene QuikChange site specific mutagenesis kit (Stratagene, Inc., La Jolla, CA) was used to introduce the restriction site with the following mutagenic oligonucleotides (*Nde*I sites are underlined): 5'-GAGGAGAAAGGTACATATGGGTGAACAGAAAC-3' (SEQ ID NO:7) and 5'-CAGTTTCTGTTCACCCATATGTACCTTTCTCC-3' (SEQ ID NO:8).

The spinach lycopene ε cyclase was cloned into the *Nde*I site of pPRONde to produce pPRONde-LEC, which was used to transform electrocompetent *P. stewartii* as described above. As a negative control, *P. stewartii* also were transformed with "empty" pPRONde (i.e., without the spinach lycopene ε cyclase gene). Transformed *P. stewartii* were cultured as described above, except that expression was induced with arabinose and IPTG in certain samples. Carotenoids were extracted and detected by HPLC analysis as described above. Lutein eluted at approximately 6.62 minutes and zeaxanthin eluted at approximately 7.27 minutes. Panel A of FIG 5 contains chromatograms of lutein and zeaxanthin standards.

An increase in lutein production was observed in *P. stewartii* cells transformed with pPRONde-LEC and induced with arabinose and IPTG (Panel B of FIG 5). There was minimal lutein production in *P. stewartii* cells transformed with pPRONde-LEC (Panel C of FIG 5) and cultured under normal conditions (i.e., not induced). No lutein

production was observed in *P. stewartii* transformed with empty vector under normal conditions or after induction with arabinose and IPTG (Panels D and E of FIG 5, respectively).

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid having at least 75% sequence identity to the nucleotide sequence of nucleotides 264-1817 of SEQ ID NO:5 or to a fragment of the nucleic acid of SEQ ID NO:5 at least 34 contiguous nucleotides in length.

2. The nucleic acid of claim 1, said nucleic acid having at least 80% sequence identity to the nucleotide sequence of SEQ ID NO:5 or to said fragment.

3. The nucleic acid of claim 1, said nucleic acid having at least 85% sequence identity to the nucleotide sequence of SEQ ID NO:5 or to said fragment.

4. The nucleic acid of claim 1, said nucleic acid having at least 90% sequence identity to the nucleotide sequence of SEQ ID NO:5 or to said fragment.

5. The nucleic acid of claim 1, said nucleic acid having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:5 or to said fragment.

6. An expression vector comprising the nucleic acid of claim 1 operably linked to an expression control element.

7. An isolated nucleic acid encoding a lycopene ε cyclase polypeptide at least 75% identical to the amino acid sequence of SEQ ID NO:6.

8. An isolated polypeptide having at least 85% sequence identity to the amino acid sequence of SEQ ID NO:6.

9. The isolated polypeptide of claim 8, said polypeptide having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:6.

10. The isolated polypeptide of claim 8, said polypeptide having at least 99% sequence identity to the amino acid sequence of SEQ ID NO:6.

11. A microorganism, wherein said microorganism comprises an exogenous nucleic acid encoding a lycopene ε cyclase, and wherein said microorganism produces detectable amounts of lutein.

12. The microorganism of claim 11, wherein α-carotene is converted to lutein in said microorganism by a single polypeptide.

13. The microorganism of claim 11, wherein said microorganism naturally produces β-carotene or hydroxylated derivatives of β-carotene.

14. The microorganism of claim 11, wherein said microorganism naturally produces zeaxanthin.

15. The microorganism of claim 11, wherein said microorganism naturally produces lycopene.

16. The microorganism of claim 12, wherein said polypeptide is endogenous to said microorganism.

17. The microorganism of claim 12, wherein said polypeptide is exogenous to said microorganism.

18. The microorganism of claim 11, wherein said microorganism is a species from a genus selected from the group consisting of *Pantoea*, *Escherichia*, and *Rhodobacter*.

19. The microorganism of claim 18, wherein said *Rhodobacter* species is *R. sphaeroides* or *R. capsulatus*.

20. The microorganism of claim 18, wherein said *Escherichia* species is *E. vulneris*.

21. The microorganism of claim 18, wherein said *Pantoea* species is *P. stewartii*.

22. The microorganism of claim 11, wherein said microorganism is a membranous bacteria.

23. A microorganism comprising biosynthetic enzymes, wherein said biosynthetic enzymes are geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene dehydrogenase, lycopene β-cyclase, lycopene ε cyclase, and β-carotene hydroxylase, wherein each of said biosynthetic enzymes is encoded by an exogenous nucleic acid, wherein expression of said biosynthetic enzymes in said microorganism produces lutein, and wherein said microorganism does not naturally produce carotenoids.

24. A microorganism that produces detectable amounts of zeaxanthin and lutein, wherein said microorganism comprises a first exogenous nucleic acid encoding a lycopene ε cyclase operably linked to a first inducible expression control element, wherein α-carotene is converted to lutein in said microorganism by a single polypeptide, and wherein inducible expression of said first exogenous nucleic acid regulates the ratio of zeaxanthin to lutein in said microorganism.

25. The microorganism of claim 24, wherein said microorganism comprises a second exogenous nucleic acid encoding a lycopene β cyclase operably linked to a second inducible expression control element, wherein inducible expression of said first and said second exogenous nucleic acids regulates the ratio of zeaxanthin to lutein in said microorganism.

26. A method of producing lutein, said method comprising expressing lycopene ε cyclase in a microorganism, wherein said microorganism comprises an exogenous nucleic acid encoding said lycopene ε cyclase; and extracting lutein from said microorganism.

27. The method of claim 26, wherein α-carotene is converted to lutein in said microorganism by a single polypeptide.

28. A method of producing a feed supplement, said method comprising expressing lycopene ε cyclase in a microorganism, wherein said microorganism comprises an exogenous nucleic acid encoding said lycopene ε cyclase; and drying said microorganism to produce said feed supplement.

29. The method of claim 28, wherein α-carotene is converted to lutein in said microorganism by a single polypeptide.

30. A method of producing an enhanced food product, said method comprising expressing lycopene ε cyclase in a microorganism, wherein said microorganism comprises an exogenous nucleic acid encoding said lycopene ε cyclase; extracting lutein from said microorganism; and adding said lutein to a food product to obtain said enhanced food product.

31. An algal cell, wherein said algal cell comprises an exogenous nucleic acid encoding a lycopene ε cyclase, and wherein said algal cell produces detectable amounts of lutein.

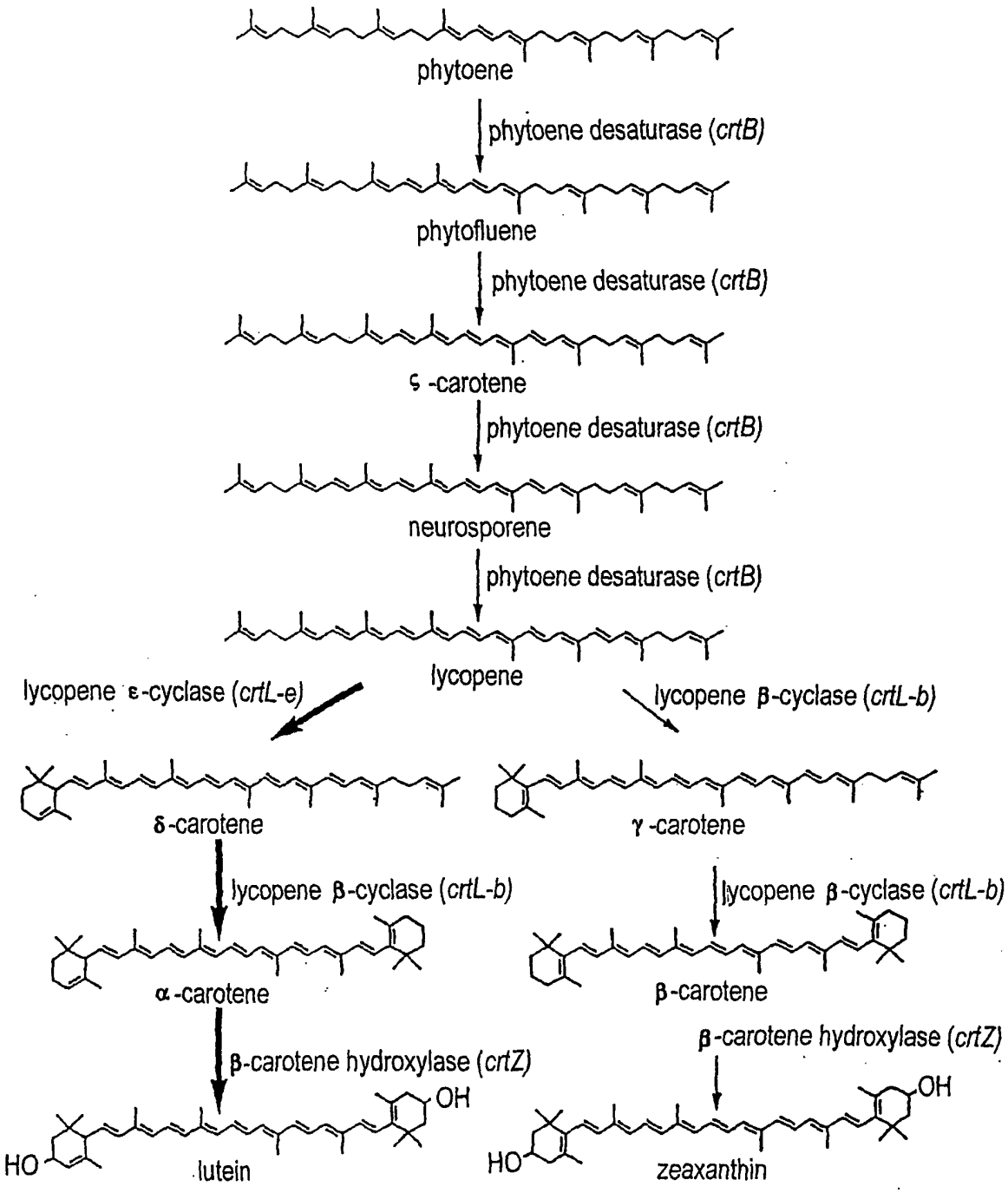
phytoene
phytoene desaturase (crtB)
phytofluene
phytoene desaturase (crtB)
ς -carotene
phytoene desaturase (crtB)
neurosporene
phytoene desaturase (crtB)
lycopene
lycopene ε-cyclase (crtL-e)
lycopene β-cyclase (crtL-b)
δ-carotene
γ -carotene
lycopene β-cyclase (crtL-b)
lycopene β-cyclase (crtL-b)
α -carotene
β-carotene
β-carotene hydroxylase (crtZ)
β-carotene hydroxylase (crtZ)
OH
HO
lutein
OH
HO
zeaxanthin

FIG. 1

```
   1  GCACGAGACA CCACAAAACC ATTGAGGAGA GAGAAAGTCA ACCAAATTTC
  51  ACACCTTCCA CCTCCCTCTT CCATGGCCGC AACCCAAACC CAGCCACCTT
 101  CACCGCCGCC GTCGACAGCA CACTGAACTT CACCACTACA AACTTAAAAA
 151  AAATCTTGGA GAAATTTGAT TCCGTAAAAA TGGAGTATTA TTGTCTCGGA
 201  GCTTCGAAAT TCGCAACAAT GGCGGTTTCT CCTGCGCTTA ATCACGACAA
 251  TTTGAGGAAT AAAATGGTTA AACAACGCCA GAATTTCCAG ACGTTTTGCT
 301  TTTGGAGGCC GAATTCTTCG AACGTTGTAG TAGAATGTAG TAGTCGTAGG
 351  AGTGGAAGTA GTGTTTTGAG GAGTGCGAAT AGCGACAGTA GTTGCGTAAT
 401  TGCGCCAGAG GATTTTGCGA ACGAAGAAGA TTTCATCAAA GCTGGTGGTT
 451  CCGAGCTTCT TTATGTTCAA ATGCAGCAGA ATAAAGCTAT GGATTGTTAC
 501  TCCAAAATTT CCGATAAGCT GCGTCAAATA TCAGATGCCA ATGAACTGCT
 551  GGATATGGTG GTTATTGGTT GTGGTCCAGC TGGTCTAGCT TTGGCTGCAG
 601  AATCGGCTAA ACTTGGATTA AAAGTTGGCC TTGTTGGTCC TGATCTTCCT
 651  TTTACGAATA ACTACGGCGT TTGGGAAGAT GAATTCAGAG CATTGGGACT
 701  TGGAGGCTGT ATCGAGCACG TTTGGCGTGA TACCATTGTG TATATTGATG
 751  ATGACAATCC TATATATATT GGTCGATCTT ATGGAAAAGT CAGCCGGCAA
 801  TTACTTCACA AGGAACTGGT GCACAGGTGT TTGGAGTCAG GTGTCTCTTA
 851  TCTGAATGCG AAAGTGGAAA ATATTATGGA AGGACCTGAT GGACATAGGC
 901  TTGTTGCTTG TGAACGTGGT GTCACTATTC CCTGCAGGCT TGTAACTGTT
 951  GCATCTGGAG CAGCTTCAGG GAAACTTCTG GAGTATGAAG TGGGTGGTCC
1001  AAGGGTTTGT GTACAAACAG CTTATGGTGT GGAGGTGGAG GTGGAAAACA
1051  GTCCTTATGA TCCCAATGTG ATGGTGTTCA TGGACTACAG AGACTACACT
1101  AAACTGAGCG TTCAATCTCT GGAGGCAAAG TATCCAACAT TCTTGTATGC
1151  AATGCCGATA TCACCAACTA GGATCTTCTT TGAGGAGACT TGCTTGGCTT
1201  CAGTAGATGC AATGCCCTTT GACCTGCTCA AGAAAAAGCT TATGACAAGA
1251  TTACAAACTA TGGGTGTTCG TATCACCAAA ATATATGAAG AGGAGTGGTC
1301  TTATATACCT GTTGGTGGGT CCTTACCAAA TACAGAGCAA AGAAACCTTG
1351  CATTTGGTGC TGCTGCGAGC ATGGTGCATC CAGCCACAGG TTATTCAGTC
```

FIG. 2A

1401 GTGAGATCAC TGTCAGAAGC TCCAAAGTAT GCTTCTGCAA TTGCAAACTT

1451 GATCAAGAAT GACCTGTCAA AAAATGCAAT ATTGCGTCAG AGGAGTGTGG

1501 GGAATATCTC AATGCAAGCC TGGAATACTC TTTGGCCACA AGAAAGGAAA

1551 CGTCAGAGAG CATTCTTCCT GTTCGGACTA TCACTTATAG TCCAGCTTGA

1601 TATTGAGGGT ATCAGGACAT TCTTCCGCAC CTTCTTCCGA GTGCCAAAAT

1651 GGATGTGGGA GGGATTCCTC GGTTCTAATC TCTCTTCAGC TGATCTCATA

1701 TTGTTTGCCT TTTATATGTT CTTTATTGCT CCGAATGACT TGAGAATGGG

1751 TCTTATAAGG CATCTACTAT CTGATCCTAC AGGGGCGACC ATGATAAGAA

1801 CGTACATAAC ACTA**TAA**AAG TAATATGAAA TGCTCACTCC TTTGTACATC

1851 ATGCAAAATT GGTACGAATT GACTGGACTA TGCAGTCTAA CTTGGTGTAA

1901 AAAAAACACA ATTAATAAAT TTTTTGTAGG TGCAGCCTCT ATACTTGATA

1951 TTCTCGATTC AGATATAATA TTGTCAGTAT TCTTCGTTAA AGATCAGTTG

2001 TTTCTACAAT TCCAGAGGCT CCTGGAATTG GTGTTACCCT TCCATGTAGC

2051 TCATTGATAA ATGTTGAGGG TAGAGGCTTT TTCTTAGATG CTTGCTTGCA

2101 GCTTGCTCAT GGATATATTC AGTTGTTCAG TAGACACGTT AACAACTACT

2151 ACAGTGGGGG CATCATTGAT CTGGACCGGG AGAGCTGAGC ATCTATCACA

2201 GGTTAGCCAG CTCAACTACG TAGGTCAACC TTGAGCCACT CCCAAACATT

2251 TTTGCAGCTG ATGGGGTTCA CCCTGTAAGG TGAGTTTCTT ACCAACTCCA

2301 CCAACTTATG TTGGTTTTAA ATTGCTACTC GTCTGTTATG AAGTAGCAAG

2351 CTCGTGCCGA ATTCCTGCAG CCCGGGGGAT CCACTAGTTC TAGAGCGGCC

2401 GCCACCGCGG TGGAGCTCCA GCTTTT

FIG. 2B

```
  1  MVKQRQNFQT FCFWRPNSSN VVVECSSRRS GSSVLRSANS DSSCVIAPED
 51  FANEEDFIKA GGSELLYVQM QQNKAMDCYS KISDKLRQIS DANELLDMVV
101  IGCGPAGLAL AAESAKLGLK VGLVGPDLPF TNNYGVWEDE FRALGLGGCI
151  EHVWRDTIVY IDDDNPIYIG RSYGKVSRQL LHKELVHRCL ESGVSYLNAK
201  VENIMEGPDG HRLVACERGV TIPCRLVTVA SGAASGKLLE YEVGGPRVCV
251  QTAYGVEVEV ENSPYDPNVM VFMDYRDYTK LSVQSLEAKY PTFLYAMPIS
301  PTRIFFEETC LASVDAMPFD LLKKKLMTRL QTMGVRITKI YEEEWSYIPV
351  GGSLPNTEQR NLAFGAAASM VHPATGYSVV RSLSEAPKYA SAIANLIKND
401  LSKNAILRQR SVGNISMQAW NTLWPQERKR QRAFFLFGLS LIVQLDIEGI
451  RTFFRTFFRV PKWMWEGFLG SNLSSADLIL FAFYMFFIAP NDLRMGLIRH
501  LLSDPTGATM IRTYITL
```

FIG. 3

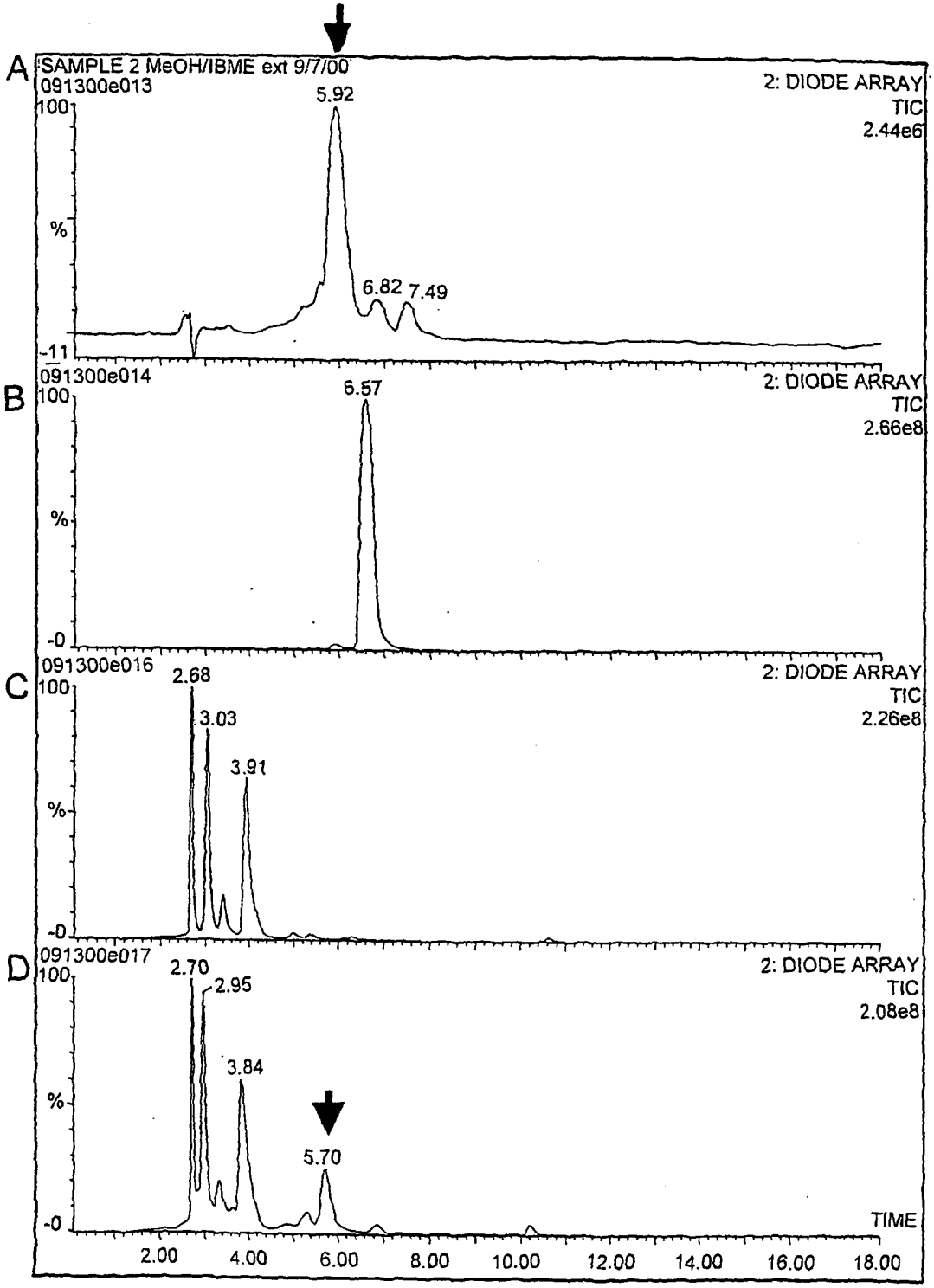
A
SAMPLE 2 MeOH/IBME ext 9/7/00
091300e013
2: DIODE ARRAY
TIC
2.44e6
5.92
6.82
7.49
100
%
-11
B
091300e014
2: DIODE ARRAY
TIC
2.66e8
6.57
100
%
-0
C
091300e016
2: DIODE ARRAY
TIC
2.26e8
2.68
3.03
3.91
100
%
-0
D
091300e017
2: DIODE ARRAY
TIC
2.08e8
2.70
2.95
3.84
5.70
100
%
-0
TIME
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00

FIG. 4

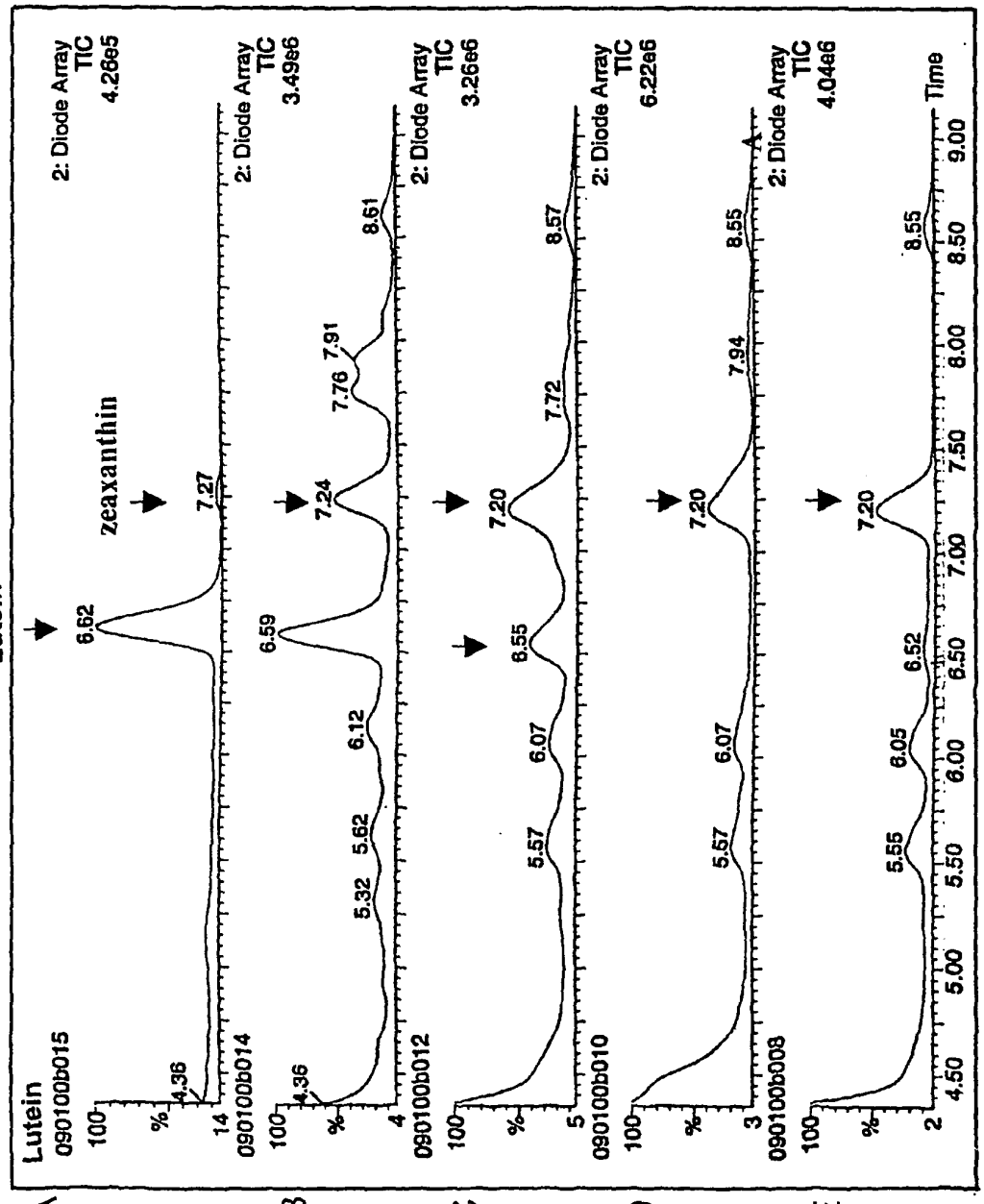
Lutein
zeaxanthin
A
B
C
D
E
Lutein
090100b015
2: Diode Array
TIC
4.26e5
090100b014
3.49e6
090100b012
3.26e6
090100b010
6.22e6
090100b008
4.04e6
6.62
7.27
4.36
6.59
7.24
7.76
7.91
8.61
5.32
5.62
6.12
7.20
5.57
6.07
8.55
7.72
8.57
7.94
8.55
5.55
6.05
6.52
Time

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/07178

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 9/02, 1/20, 15/63; C07H 21/04

US CL :435/189, 252.3, 320.1; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/189, 252.3, 320.1; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

West searches for US Patents, EPO, JPO and Derwent; STN files included - Medline, Caplus, Biosis, Biotechds, Emabase and Scisearch.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,744,341 A (CUNNINGHAM, Jr. et al.) 28 April, 1998, see abstract and examples, columns 7-12.	1-10
A	CUNNINGHAM, F. X., Jr. et al. Functional Analysis of the β and ε Lycopene Cyclase Enzymes of Arabidopsis Reveals a Mechanism for Control of Cyclic Carotenoid Formation. The Plant Cell. September 1996, Vol. 8, pages 1613-1626.	1-10
A	MATSUMURA, H. et al. Cloning, sequencing and expressing the carotenoid biosynthesis genes, lycopene cyclase and phytoene desaturase, from the aerobic photosynthetic bacterium Erythrobacter longus sp. strain Och 101 in Escherichia coli. Gene. 21 April 1997, Vol. 189, pages 169-174.	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
19 JUNE 2001	12 JUL 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer TEKCHAND SAIDHA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/07178

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/07178

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-10, drawn to isolated nucleic acid of SEQ ID NO : 5 encoding the polypeptide of SEQ ID NO : 6 (lycopene epsilon cyclase) and expression vector comprising the nucleic acid of SEQ ID NO : 5.
Group II, claim(s) 11-22, 26-29 drawn to a transformed microorganism comprising the nucleic acid encoding lycopene cyclase.
Group III, claim(s) 23-25, drawn to a transformed microorganism encoding multiple genes of the carotenoid biosynthesis.
Group IV, claim(s)30-31, drawn to method of producing food product.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I has a special technical feature of nucleotide sequence of SEQ ID NO : 5 and the amino acid sequence of SEQ ID NO : 6, which groups II-III do not share. Further, the prior art reveals transformed host cells containing gene encoding lycopene cyclase and the pathway leading to lutein [The Plant Cell 8 : 1613-26, 1996]. With the gene and the encoded enzyme known, the invention of Group I and II or Group I & III, lack the special technical feature and therefore lack unity of invention. Group II has a special technical feature of a transformed microorganism, encoding any lycopene cyclase, which is known in the art cited and therefore, lack unity of invention with Groups I and III-IV. Group III has a special technical feature of a transformed microorganism, encoding multiple genes of the carotenoid biosynthetic pathway, which Groups I-II and IV do not share. Group VI has a special technical feature of a method of producing food product which Groups I-III do not share. Thus the various groups discussed show a lack of unity of invention.